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PAPER

Separation of bacteria with imprinted polymeric films†

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Separation of compounds out of complex mixtures is a key issue that has been solved for small molecules by chromatography. However, general methods for the separation of large bio-particles, such as cells, are still challenging. We demonstrate integration of imprinted polymeric films (IPF) into a microfluidic chip, which preferentially capture cells matching an imprint template, and separate strains of cyanobacteria with 80–90% efficiency, despite a minimal difference in morphology and fluorescence, demonstrating its general nature. It is currently thought that the imprinting process, conducted while the polymer cures, transfers chemical information of the cell's external structure to the substrate. Capture specificity and separation can be further enhanced by orienting the imprints parallel to the flow vector and tuning the pH to a lower range.

Introduction

Separation is a key issue in many fields and applications, including analytical chemistry, diagnostics, environmental science, and synthesis and purification. In contrast to separations of small molecules, which can be performed reliably *via* chromatography, cell separations are still challenging. Filter-based^{1–3} and magnetic separations^{4,5} are the methods of choice to handle multiple cells simultaneously; however, they require that cells possess a significant size difference or be magnetically labelled.

Molecularly imprinted polymers are widely used as stationary phases in chromatography,^{6–9} sensors,^{10,11} platforms for drug delivery,^{12,13} as well as artificial enzymes.^{14–16} They are relatively cheap and easy to produce, and the high number of commercially available monomers allows for properties to be tuned for different analytes.¹⁷ However, large bio-particles such as proteins,^{18,19} viruses²⁰ and entire cells^{21,22} cannot diffuse through a bulk imprinted material. The use of a molecularly imprinted polymer film (IPF) circumvents this problem, but exposure of the particles to the stationary phase is limited to only the surface of the film. Microfluidics offers an attractive solution to this problem, as the surface-to-volume ratio of such architectures is dramatically increased compared to macroscale techniques, diffusion distances are decreased, and the IPF may be integrated with other analytical techniques on the same microdevice. However, IPFs incorporated into microfluidic platforms have, as of yet, only been applied to bio-particles such as proteins²³ and viruses;²⁴ not cells. A recent review of this topic may be found elsewhere.²⁵

IPFs are imprinted with templates of the target cell, forming complexes between the polymer and the analyte *via* self-assembly.^{26,27} It is theorized that the electrostatic interactions between the polymer and the cell surface continue through the cross-linking process, effectively molding the cell surface's chemical information into the polymer's exposed functional groups. When the cells are removed after the polymer substrate has been cured, they leave behind imprints that can recognize and reincorporate other cells of that template.

We demonstrate here the first use of microfluidic IPF devices for effective and specific cell separation. The effectiveness of this technique may be further enhanced by sequential separations, adjustment of pH, and the use of oriented imprints. The general nature of this technique, essentially matching a template's chemical fingerprint to that of cells in a sample, holds promise for use in a number of fields. In this study, we have performed all separations on strains of *Synechococcus* and *Synechocystis* cyanobacteria, which represent important organisms which arise from exceptionally diverse microenvironments,²⁸ indicating the possible application of this technique toward separation of target organisms from complex samples. We chose two related strains of *Synechococcus* to demonstrate the specificity of this sorting technique. The external layers of these cells are expected to be exceptionally diverse, a feature believed to arise from environmental factors.²⁹ We believe that the use of microfluidic IPF devices for bacterial sorting is general and can be applied to a wide range of other cell types.

Experimental details

Materials

Synechococcus OS-B' (*Syn* OS-B'), *Synechococcus elongatus* PCC 7942 (*Syn* 7942), and *Synechocystis* PCC 6803 (6803) were obtained from the laboratory of Devaki Bhaya in the

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Department of Plant Biology, Carnegie Institute of Washington. Poly(dimethylsiloxane) (PDMS) was obtained from RS Hughes. A viability test was performed using LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen) following the protocol given by the vendor. All other chemicals were purchased in highest or analytical grade from Sigma Aldrich or VWR.

Fabrication of microfluidic chips

The microfluidic chips are composed of two parts: a top PDMS layer, containing a simple serpentine microfluidic channel, and an IPF. The serpentine channel (20 μm in height, 100 μm in width, and 61.88 mm in length) was cast into the PDMS *via* standard soft lithography. The top layer was bonded to the imprinted polymer using PDMS-mortar (10 : 1 PDMS diluted in four parts toluene spin-coated on glass and pre-cured at 80 $^{\circ}\text{C}$ for 4 min). The fabrication of the imprinted polymers is described below.

Preparation of template stamps

A prerequisite for imprinting with cyanobacteria is a glass stamp with adhered cells. For this work, stamps with cells in a random or defined orientation were used. For randomly oriented cells, 5 \times 5 mm pieces of microscope slides were polished with Kimwipes (to remove dust or fingerprints) and 40 μL of cell suspension (approximately 3×10^8 cells mL^{-1}) was spread on the surface. After 30 min at 4 $^{\circ}\text{C}$, the cells had settled down on the glass plate and the surplus solvent was removed by spinning the slide at 1500 rpm (1 min) prior to drying. This step is critical to prevent crystallized buffer salts from covering cells and obstructing imprinting.

Fig. 1 illustrates how stamps with cells in a defined orientation were produced. A glass plate covered with 0.01% polylysine solution was left at room temperature for 10 min. Surplus solvent was removed on the edges with a Kimwipe, followed by drying. A directional flow was employed to promote cell placement on the surface in a defined orientation. This was achieved by

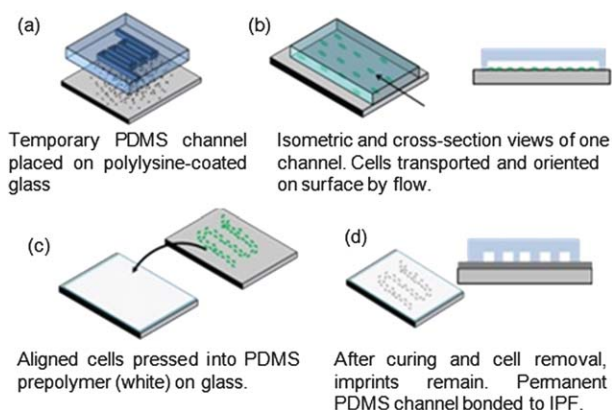


Fig. 1 Fabrication scheme for oriented IPF microdevice. (a) Temporary PDMS structure placed on polylysine-coated glass. (b) A cell suspension is moved through the channel using negative pressure, oriented by flow. (c) The top layer is removed and the glass plate with oriented cells is used for imprinting. (d) Oriented imprints remain when the cells are removed. Cross-section shows the final chip after bonding of permanent structure (100 μm channels).

temporarily binding a PDMS layer containing a serpentine channel to the glass plate, *via* simple electrostatic interaction. Consequently, the binding was not very strong and negative pressure was needed to apply a flow. A pipette tip, serving as reservoir, was connected to the inlet and a syringe was used to suck cell suspension through the chip from the outlet. During that process, the rod-shaped cells were bound to the surface, oriented with the flow vector. 50 μL , which corresponds to approximately 10^7 cells mL^{-1} , was moved through the chip. After removal of the suspension, the PDMS was peeled from the glass plate, which could then be used for imprinting.

Fabrication of molecularly imprinted films

Optimization of the imprinting protocol was conducted, and is discussed in the Supplementary Information document. Briefly, the optimal protocol was determined to be as follows. 2 parts 10 : 1 (monomer:crosslinker) PDMS was diluted with 1 part cyclohexane and spin-coated onto a microscope slide (30 s at 1500 rpm). Pre-curing at 80 $^{\circ}\text{C}$ for 4 min enhances the viscosity of the prepolymer, preventing the cells from sinking too deeply into the material. Glass stamps with adhered cells were pressed into the prepolymer and the polymer was finally cured at room temperature overnight. Alternatively, curing could also be performed at an elevated temperature. However, it is generally known that molecularly imprinted polymers are more selective if the prepolymer has more time (using a lower temperature) to form high-affinity binding sites.³⁰

Cell removal is performed by submerging the IPF in a petri dish filled with distilled water and sonicating for five minutes. The success of the imprinting process can be determined *via* AFM (Fig. 2).

Capture & separation of cyanobacteria

A pipette tip was inserted into the chip's inlet, to serve as a reservoir, and filled with cell suspension. The outlet was connected to a syringe and cell suspension was drawn through the channel *via* negative pressure. After a certain volume (specified respectively for each experiment discussed below) was passed through the device, the channels were scanned on a microscope platform and cells were counted automatically.

An image of an area of the channel was taken using a CCD camera (Mintron MTV-63KR11N) and an inverted microscope (Nikon Eclipse TE2000-U). The brightness of the pixels corresponds to photon counts on a particular area of the CCD chip. A group of pixels exceeding an empirically determined threshold was counted as a particle. A laser beam (Crysta Laser, maximum

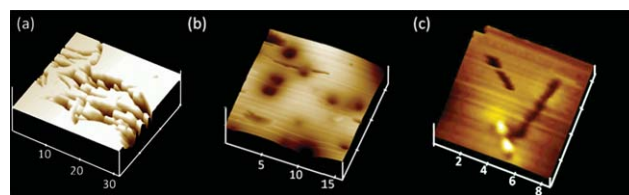


Fig. 2 AFM images of imprinted PDMS surfaces following cell removal: (a) *Synechococcus* OS-B', (b) *Synechocystis* PCC 6803, (c) *Synechococcus elongates* PCC 7942.

output power: 495 mW at 633 nm) was expanded to cover an area of approximately $400\ \mu\text{m} \times 400\ \mu\text{m}$ within the separation device, which can be regarded as the detection area. The device was placed on a precisely movable stage (Lstep Märzhäuser) which was controlled remotely. A Labview (National Instruments) program built in-house allowed us to perform a raster scan in order to cover the whole separation area systematically. Additionally, the camera control was synchronized to this scanning movement in such a way that we automatically obtained a composite fluorescence image of the entire separation area. After appropriate calibration for each type of particle, this automated scanning procedure allowed us to achieve a rapid and reliable quantification of the cells caught on the capturing surface.

The chips can be regenerated and the purified cells can be released by washing with an excess of polylysine (0.01%) aqueous solution. The oligopeptide is positively charged and competes with the positive charges on the IPF, causing release of the cells. Three capture and release cycles with *Syn OS-B'* are shown in Fig. 3. Separation capability of the microdevice was first tested and optimized with fluorescent beads and *Syn OS-B'* (Supplementary Information) and a separation efficiency of $90 \pm 4\%$ at a flow rate of about $20\ \text{mL min}^{-1}$ was achieved.

Results and discussion

Cell samples can be significantly enriched on the surface by flushing a cell suspension through the chip. Fig. 4 shows an example of *Syn OS-B'* enrichment on a polylysine-coated surface imprinted with a *Syn OS-B'* template; saturation occurs after the chip has processed a given volume.

The specificity of IPF capture was evaluated by comparing the capture of the three cell strains across five types of surfaces. It can be seen in Fig. 5 that each imprinted film incorporates significantly more cells of its template than non-template cells. This specificity is especially striking between the two strains of *Synechococcus* cyanobacteria, *Syn 7942* and *Syn OS-B'*. Bare PDMS and non-imprinted polylysine-coated glass demonstrate no such specificity.

The separation efficiency that results from this capture specificity was also evaluated. A 1 : 1 mixture of *Syn OS-B'* and 6803

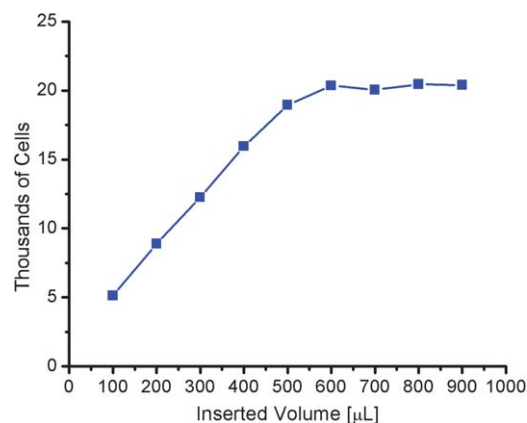


Fig. 4 Accumulation of *Syn OS-B'* cells on an imprinted surface.

($10^5\ \text{cells mL}^{-1}$) was used. The mixture was processed through one of two separate microdevices: one with a 6803-imprinted surface and another with a *Syn OS-B'*-imprinted surface. Following processing, the resulting suspensions, now depleted in cells matching the imprint template, were analyzed by flow cytometry. The efficacy of sequential separations was examined by processing suspensions through several new chips with the same type of IPF. Fig. 6 shows that processing through one device of either IPF type results in a separation efficiency of about 80%, while processing through sequential devices of the same type can improve the efficiency up to about 90%.

Fig. 7 shows the sensor response of a surface imprinted with *Syn OS-B'*, and two non-imprinted references: bare PDMS and polylysine-coated glass. The imprinted surface exhibits much greater sensitivity than both non-imprinted references. We believe that this indicates promising potential for microfluidic IPF devices to be used in bacterial detection applications, such as medical diagnostics or characterization of environmental bacterial populations.

Adhesion of cells to imprinted surfaces, and thus the sensitivity and efficiency of capture and separation, can be further enhanced by adjusting the pH of the suspension. Suspensions of *Syn OS-B'* and *Syn 7942* were processed through chips with *Syn OS-B'*-imprinted IPFs, as well as a chip with a non-imprinted reference

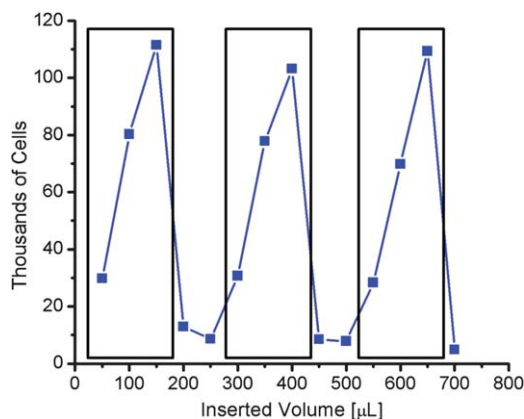


Fig. 3 Reversibility of *Syn OS-B'* capture. When a cell suspension is injected, cells are accumulated on the surface (black frames). After each accumulation, the chip was washed with 0.01% polylysine in water.

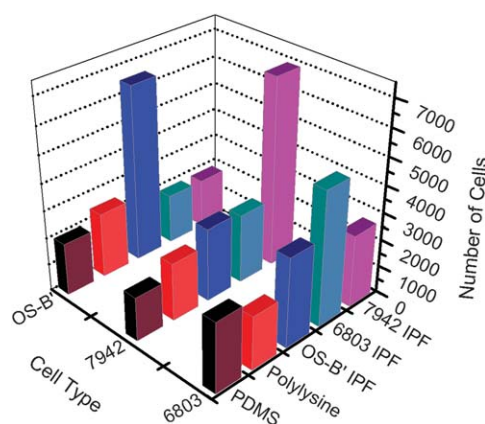


Fig. 5 Selectivities of different coatings (y-axis) exposed to the same concentration of respective bacterial species (x-axis).

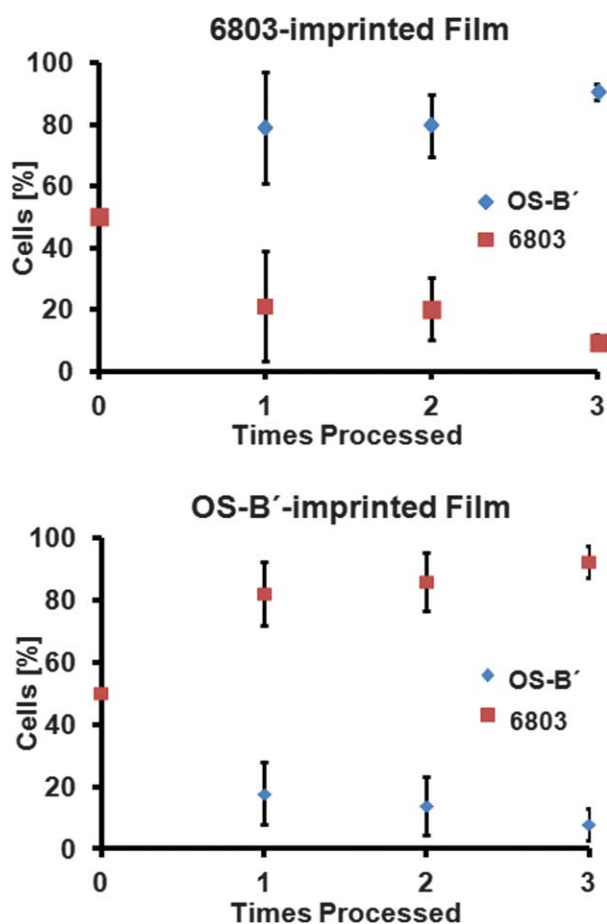


Fig. 6 1 : 1 mixtures of two cyanobacterial strains (*Syn* OS-B' and 6803) were processed sequentially through separate devices of two types: 6803-imprinted (top) and *Syn* OS-B'-imprinted (bottom). Flow cytometry data was taken of each suspension after processing through each device.

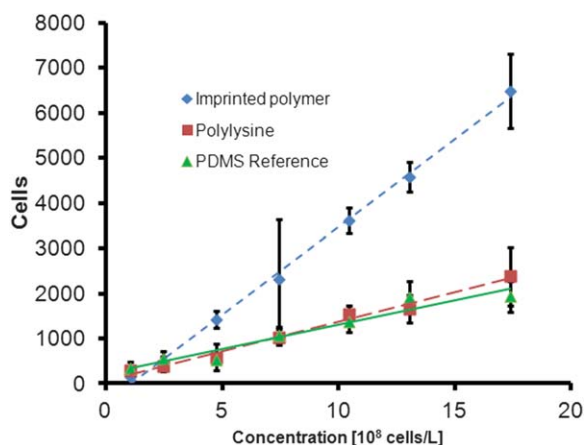


Fig. 7 Sensor responses of *Syn* OS-B'-imprinted PDMS, polylysine, and bare PDMS to a range of *Syn* OS-B' concentrations.

(Fig. 8). pH was tuned using either KOH, acetic acid or HCl, and no significant difference was observed between the effects of the additives (data not shown). At a sufficiently low value (pH 4), more cells of both types were captured than at pH 7. However, there is exists an optimal value (pH 5) where selectivity greatly

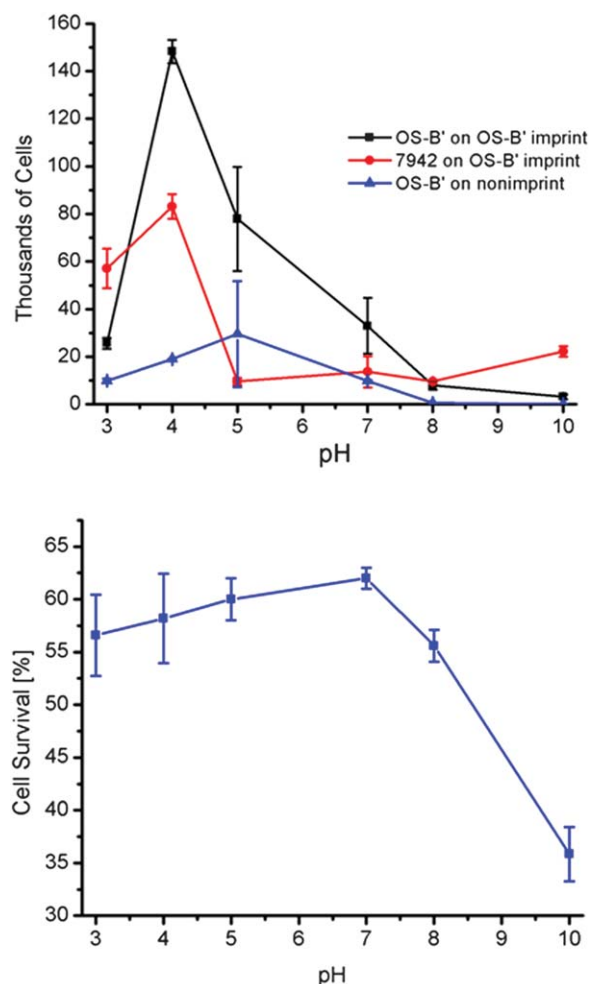


Fig. 8 The adhesion of *Syn* OS-B' and *Syn* 7942 to *Syn* OS-B'-imprinted surfaces and a non-imprinted reference at different pH values was measured (top). Additionally, viability of *Syn* OS-B' under the same range of pH values was also studied. The viability of an untreated reference was $63 \pm 4\%$. Error bars were determined from three experiments using different chips.

enhanced, as more *Syn* OS-B' cells are captured while 'cross-capture' of *Syn* 7942 is actually reduced. We believe that this effect is explained by protonation of functional groups, both on the surfaces of the cells and the imprints, leading to a more favourable electrostatic interaction between the two. Notably, viability of cells that are captured and then flushed from the device changes only slightly over the range of this pH (Fig. 8, bottom), from the value of $63 \pm 4\%$ on a non-imprinted reference at pH 7.

Further enhancement to capture efficiency and sensitivity can be achieved by creating an IPF where the imprints are in a single defined orientation. Imprints oriented parallel to the flow, perpendicular to the flow, and at random were tested against a non-imprinted PDMS reference (Fig. 9). Imprints oriented parallel to the flow show an enhanced capturing efficiency compared to imprints oriented perpendicular to the flow or at random, though all imprint directions are still more sensitive than a non-imprinted surface. Additionally, pH adjustment can be used to enhance capture even further.

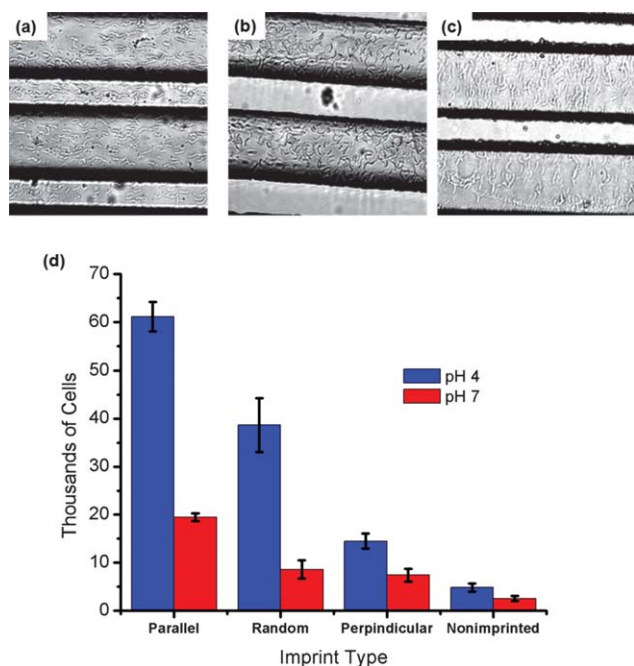


Fig. 9 Comparison of imprints oriented (a) parallel to the flow, (b) at random, and (c) perpendicular to the flow. Capture efficiency of each imprint type, as well as a non-imprinted reference, to *Syn OS-B'* was tested at pH 4 and pH 7 (d).

Conclusions

We have demonstrated the first use of microfluidic IPF devices for cell capture and separation, as well as the first use of oriented imprints. We have demonstrated that microfluidic IPFs possess a high specificity for template cells, even when a suspension contains species having very similar morphologies that cannot be well-distinguished by size-based methods. When separating different strains of cyanobacteria, separation efficiencies between 80% and 90% were achieved, depending on the number of sequential separations employed. Additionally, we demonstrated that microfluidic IPFs have a dynamic sensor response, indicating their potential use for bacterial detection applications.

The initial achievements in separation efficiency and cell capture were demonstrated to be enhanced *via* several techniques. In addition to simple additional processing in sequence with the same microfluidic IPF type, separation could also be improved by adjusting the pH to a more acidic value and orienting the imprints parallel to the flow direction. As it is currently thought that molecular imprinting transfers chemical information of a cell's external architecture to the polymer, these methods hold promise as an effective, general method for cell separation that does not require significant differences in morphology or labels (magnetic, fluorescent, or otherwise).

While our work here with cyanobacteria demonstrates this technique has potential for working with bacteria that typically arise from diverse microenvironments, we believe this technique may also have important uses in medical diagnostics, such as detection of one or a mix of suspected agents from blood or urine samples. The power of this technique arises from its general nature, relying purely on the chemical fingerprint of a cell's surface.

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